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Anti-*Pseudomonas* activity of 3-nitro-4-phenylfuroxan

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18 **Abbreviations**

19 MGE (mobile genetic elements), GFP (green fluorescent protein), NO (nitric oxide), MIC (minimal
20 inhibitory concentration), CLSI (Clinical and Laboratory Standard Institute), LB (Luria Bertani), CV
21 (crystal violet), CLSM (Confocal Laser Scanning Microscopy), UV (Ultraviolet), HPLC (High
22 pressure Liquid Chromatography), ANOVA (Analysis of Variance), DMSO (dimethyl sulfoxide).

23

24 **Abstract**

25 *Pseudomonas aeruginosa* is a microorganism that is well adapted to both clinical and industrial
26 settings, where it can form adherent communities that are difficult to eradicate. New anti-
27 *Pseudomonas* compounds and strategies are necessary, as the current antimicrobial approaches for
28 the inhibition of biofilm formation and, above all, the eradication of formed biofilms are ineffective.

29 Compounds that belong to the furoxan-family, which are well-known NO donors, have recently
30 been shown to display anti-*Pseudomonas* activity. The present study investigates three furoxan
31 compounds that are substituted at the heteroring with electron-withdrawing groups (NO₂, CN,
32 CONH₂) for their effects on *P. aeruginosa* PAO1 growth and biofilm formation/dispersal. Of the
33 furoxans tested, only 3-nitro-4-phenylfuroxan (KN455) inhibited the growth of suspended *P.*
34 *aeruginosa* PAO1 cultures. Furthermore, KN455 inhibited the formation of both younger and older
35 biofilms with very high yields and thus proved itself to be toxic to planktonic subpopulations. It also
36 displayed moderate eradicating power. The activity of KN455 does not appear to be related to its
37 capacity to release small amounts of NO. Interestingly, the isomer 4-nitro-3-phenylfuroxan (KN454),
38 included for comparison, displayed a comparable antibiofilm rate, but did not show the same
39 antimicrobial activity against suspended cells and planktonic subpopulations. While hypotheses as to
40 the mechanism of action have been formulated, further investigations are necessary to shed light onto
41 the antimicrobial activity of this furoxan.

42

43

44 **Introduction**

45 *Pseudomonas aeruginosa* is a microorganism that is optimally adapted to a variety of
46 environmental conditions and is ubiquitously present in soil, water and sewage as well as in human,
47 animal and plant hosts [1]. *P. aeruginosa* is also known to be a human opportunistic pathogen as it
48 plays an important role in infections such as pneumonia in cystic fibrosis patients, chronic-wound
49 infections and medical-device-related infections [2, 3].

50 Treatment of *P. aeruginosa* infections is often a challenge due to its resistance to antibiotics,
51 which is acquired via a number of mechanisms. *P. aeruginosa* secretes enzymes, including β -
52 lactamases and aminoglycoside modifying enzymes, that inactivate antibiotic molecules. It can also
53 reduce antimicrobial agent accumulation in the cell producing multidrug efflux pumps (i.e. MexAB-
54 OprM, MexEF-OprN, MexCD-OprJ, MexXY-OprM, etc.) [4]. Chromosomal mutations in DNA
55 gyrase and topoisomerase IV genes lead to resistance towards quinolones [5]. The isolation of
56 multidrug-resistant strains is increasing in clinical settings, where antibiotic selection is routinely
57 practiced. Furthermore, the transfer of mobile genetic elements (MGE) contributes to the spread of
58 resistance determinants among strains that belong to the same species and those of different species
59 [6]. *P. aeruginosa* is also able to contaminate disinfectants such as chlorhexidine, benzalkonium and
60 triclosan [7].

61 The treatment of *P. aeruginosa* infections becomes more difficult when bacteria are encased in
62 their self-made matrix. This tolerance is caused by restricted compound penetration through the
63 exopolysaccharide matrix and decreased metabolism, which is due to low nutrient and oxygen levels
64 and accumulated waste [8]. The inhibition of biofilm formation and the eradication of mature biofilms
65 are goals that are important for a wide range of antimicrobial applications because microbial biofilms
66 are not only a significant issue in clinical infections, but also in the biofouling of industry water
67 systems. Anti-biofilm strategies either attempt to destroy the established community in its
68 microenvironment or target a specific step within the biofilm cycle. Biofilm disruption and/or
69 corrosion can be achieved by metal chelators, such as EGTA and EDTA [9], by surfactants, such as
70 SDS [10], by D-amino acids [11], and, as found more recently, by photoantimicrobials [12,13].

71 Nitric oxide (NO) has recently emerged as an endogenous regulator of biofilms [14]. Low
72 levels of NO induce a transition from the biofilm mode of growth to the planktonic form [15]. A
73 number of NO donors, products that can release NO under physiological conditions, have been found
74 to suppress biofilm formation and elicit dispersal events in *P. aeruginosa* [15, 16, 17]. Furoxans

(1,2,5-oxadiazole N-oxides) are an interesting class of NO donors. A recent study has described the effects of three furoxan derivatives that are able to spontaneously release nitrogen oxide species (NO_x), namely NO and nitrite (NO₂⁻), on *P. aeruginosa* PAO1 biofilm formation and dispersion [18]. The present work extends our research into the use of furoxan derivatives as potential PAO1 antibiofilm tools, and focuses on the impairment of *P. aeruginosa* biofilms using furoxan derivatives, which release NO under the action of thiol cofactors. Phenylfuroxan derivatives with electron withdrawing substituents (NO₂, CN, CONH₂) at the 3 position of the heteroring (Figure 1) are investigated for their effect on *P. aeruginosa* biofilm formation in this work, as electron-withdrawing groups have been shown to facilitate the release of NO from furoxans [19, 20]. 3-Nitro-4-phenylfuroxan (KN455), 3-cyano-4-phenylfuroxan (CF357) and 4-phenyl-3-furoxancarboxamide (CF1656) have been selected for this study. The isomer 4-nitro-3-phenylfuroxan (KN454) is included for comparison, as is the related des-NO furazan, 3-nitro-4-phenylfurazan (CF389).

Materials and methods

Synthesis of the tested compounds

Compounds KN455 [21]), CF357 [22], CF1656 [23], CF389 and KN454 [24] were synthesized as previously described.

Bacteria strains and growth conditions

Pseudomonas aeruginosa PAO1 was chosen as the model biofilm microorganism [25]. PAO1 was transformed using the pVO(GFP) expression vector; it is a pJB3 KmD derivative, in which the Green Fluorescent Protein (GFP) coding sequence was cloned under an arabinose inducible promoter (pAra). Both strains were grown overnight in LB medium at 37°C under 200 rpm shaking.

Antimicrobial assays

A modified disk diffusion assay (Kirby-Bauer test) was set up to test the sensitivity that *P. aeruginosa* PAO1 shows towards furoxans [26]. Microorganisms grown in LB overnight at 37°C were diluted 100-fold in phosphate buffer (KH₂PO₄/K₂HPO₄ 10 mM, pH 7.4) and a 100 µl sample was plated onto LB Agar. Filter paper disks (bioMerieux) with 6 mm diameters were loaded with increasing volumes of furoxans/furazan (20, 15, 10 and 5 µl) at a concentration of 50 mM, and placed on LB Agar. A disk loaded with 20 µl DMSO was used as a control. The plates were incubated at 37°C for 24 h and the antibacterial effect was evaluated by measuring the growth inhibition halo.

104 In order to evaluate the furoxans' Minimal Inhibitory Concentration (MIC) toward *P. aeruginosa*
105 PAO1, according to guidelines from CLSI (Clinical and Laboratory Standard Institute) [27], bacteria
106 were grown overnight in LB at 37°C and diluted 100-fold to give a cellular concentration of 10⁷ CFU
107 ml⁻¹. Two-fold dilutions of KN455, CF357, CF1656, KN454 and CF389 (from 2 to 0.001 mM), were
108 added to bacterial samples. The dilution series were observed for microbial growth after 24 hours of
109 incubation at 37°C and MIC values were evaluated. MBC (Minimal Bactericidal Concentrations)
110 values were determined by re-culturing dilutions that inhibited PAO1 growth (i.e. those at or above
111 the MIC). Broth dilutions were streaked onto LB agar and incubated for 24 to 48 hours. The MBC
112 was the lowest broth dilution of furoxan/furazan that prevented *P. aeruginosa* PAO1 growth on the
113 agar plate. All experiments were performed in triplicate, at least.

114 ***Biofilm assays***

115 Overnight-PAO1 cultures were diluted 1:200 times in M9 medium that was supplemented with
116 glucose 10 mM and 0.2% w/v casamino acid, used as carbon and nitrogen sources respectively, to
117 obtain homogenous and repeatable biofilms [28, 29, 30]. One ml of the diluted culture was added to
118 each well of a 24-well polystyrene plate. The furoxans were administered at three different
119 concentrations (5, 50 and 500 µM) and dimethylsulfoxide (DMSO) 0.5 % was used as a solvent
120 control. L-cysteine 5 mM, NO scavenger 2-phenyl-4,4,5,5,-tetrahydro-1H-imidazole-3-oxide-1-oxyl
121 (PTIO) 1 mM and sodium nitrite, at 0.01, 0.1 and 0.5 mM, were added when indicated. The cells were
122 incubated at 37°C under 200 rpm shaking for 6 h to evaluate the inhibitory effect of furoxans. The
123 biofilm dispersal effect of the furoxans was observed upon administering compounds for 1 h, after 5
124 h of untreated biofilm growth [18].

125 Planktonic biomass was spectrophotometrically quantified for each well via a measurement of
126 absorbance at 600 nm, while adherent biomass was quantified via crystal violet (CV) staining. Briefly,
127 planktonic biomass was removed and collected, and wells were washed once with one ml phosphate
128 buffer. One ml of 0.1% CV was added to each well for approximately 20 min to stain the biofilm,
129 after which the CV was removed and each well washed twice with 1 ml phosphate buffer. The
130 remaining CV, which indicated the amount of biofilm present, was dissolved in acetic acid 30 % for
131 10 min. The amount of solubilized dye was spectroscopically measured at 595 nm.

132 In order to evaluate the effect of the different treatments on the cellular viability of suspended
133 and adherent populations, the planktonic phase was axenically collected and adherent cells were
134 recovered by scraping and suspended in 1 ml of phosphate buffer. Viable counts - expressed as colony
135 forming units per mL, CFU ml⁻¹, in cell suspensions and as CFU per well in adherent biomass - were

136 estimated using a plate count technique; a volume (0.01 ml) of undiluted or serially diluted samples
137 was plated onto LB Agar plates and incubated for 24 h at 37°C. All experiments were independently
138 repeated at least three times.

139 *Confocal analyses*

140 Overnight PAO1- GFP tagged cultures were diluted 1:200 times in M9 medium that was
141 supplemented with glucose 10 mM and 0.2% w/v casamino acid. Five ml of the diluted culture was
142 added to a glass coverslip and placed into a well of a 6-well polystyrene plate. Volumes of the
143 furoxans, 500 μ M, and DMSO 0.5 % were added to the bacteria and subsequently incubated at 37°C.
144 After either 24 h or 48 h of incubation, cells were induced with arabinose 1 mM to express green
145 fluorescent protein. The coverslip was placed on a microscope glass slide for the acquisition of the
146 adherent biofilm image. In order to observe the suspended bacteria, cells were placed between the
147 glass and coverslip after 24 hours of treatment with furoxan/furazan 125 μ M and DMSO 0.5 % as
148 control. All microscopy observation and image acquisition was performed on a Leica TCS SP5
149 confocal laser scanning microscope (CLSM) (Leica Microsystems, Wetzlar, Germany) equipped with
150 a detector and filter set for GFP monitoring. Images were obtained using a 63/1.3 objective lens.
151 Simulated 3D images were generated using the free, open-source software ImageJ (National Institute
152 of Health, USA).

153 *NO and NO₂⁻ detection*

154 *Nitrite measurements by Griess reaction*

155 The total release of NO_x was evaluated as nitrite (NO₂⁻) using the Griess reaction. Compounds were
156 incubated at 37°C in 50 mM phosphate buffer, pH 7.4 at 0.1 mM concentration (thus final 1% DMSO)
157 either in the absence or in the presence of L-cys at 0.1, 0.5 or 1 mM (1, 5 and 10 times mol/mol
158 excess). The presence of nitrite in the reaction mixture was determined using the Griess assay at
159 regular time intervals; 1 ml of the reaction mixture was treated with 250 μ l of the Griess reagent (4%
160 w/v sulphanilamide, 0.2 % w/v N-naphthylethylenediamine dihydrochloride, 1.47 M phosphoric
161 acid). Absorbance was measured at 540 nm on a UV spectrophotometer after 10 min at room
162 temperature (UV-2501PC, Shimadzu). A calibration curve was obtained using standard solutions of
163 sodium nitrite in a concentration range of 1-80 μ M ($r^2 > 0.99$). The yield in nitrite was expressed as
164 NO₂⁻ percentage (mol/mol, relative to the initial compound concentration) \pm SEM.

165 *NO measurements using the DAN (2,3-diaminonaphthalene) method*

166 NO release from furoxan compounds was quantified using a 2,3-diaminonaphthalene (DAN)-based
167 chemical assay. This assay is based on the immediate reaction of NO with oxygen (O₂) to form
168 dinitrogen trioxide (N₂O₃), which then reacts with non-fluorescent DAN to form the highly
169 fluorescent 2,3-naphthotriazole (NAT), which can be quantified by RP-HPLC. Compounds were
170 incubated at 37°C in 50 mM phosphate buffer, pH 7.4 at 0.1 mM concentration (then final 1% DMSO)
171 with 0.2 mM (2 times mol/mol excess) DAN, either in the absence or in the presence of L-cys at 0.5
172 mM. The presence of NAT in the reaction mixture was evaluated using HPLC analysis at fixed time
173 points, according to a previously published protocol [31]. HPLC analyses were performed using a HP
174 1200 chromatograph system (Agilent Technologies, Palo Alto, CA, USA) that was equipped with a
175 quaternary pump (model G1311A), a membrane degasser (G1322A), a UV detector, MWD (model
176 G1365D), and a fluorescence detector (model G1321A), all integrated into the system. Data analyses
177 were performed on a HP ChemStation system (Agilent Technologies). The sample was eluted on a
178 Zorbax Eclipse XDB-C18 column (150 × 4.6 mm, 5 µm; Agilent); the injection volume was 20 µl.
179 The mobile phase consisted of 65% 15 mM potassium phosphate buffer (pH 8.0) and 35% acetonitrile
180 at a flow rate of 1.00 ml min⁻¹. The fluorescence signals were obtained at excitation and emission
181 wavelengths of 355 and 460 nm, respectively (gain factor = 10). Data manipulation was performed
182 using an Agilent ChemStation. The values obtained from integrating the NAT peak were interpolated
183 into a calibration line that was prepared using NaNO₂ (in acidic conditions) as a standard. Briefly,
184 sodium nitrite standard solutions were acidified with HCl (pH 2) in the presence of an excess of DAN
185 (0.2 mM). The reaction mixture was diluted in a phosphate buffer after 10 min (at pH = 7.4 and NO
186 final concentration 1 to 80 µM), and analysed by HPLC.

187 *Statistical analyses*

188 Experiments were repeated at least three times on separate dates. Mean and SD calculations
189 were performed using Microsoft Excel 2010. Data were analysed by means of one-way ANOVA
190 (Origin_ 7.0 SR0; Origin lab) and the statistical significance of all treatments was estimated (P < 0.05
191 and P < 0.01).

192 **Results**

193 *Effect of furoxans on P. aeruginosa PAO1 growth*

194 The furoxans KN455, CF357 and CF1656, which bear different substituents on the 3 position
195 of the furoxan heteroring (Figure 1), were investigated for their effect on *P. aeruginosa* PAO1 growth,
196 both in solid and liquid media. As a first step, a Kirby-Bauer test was performed to assay the activity

197 of the three compounds. The DMSO used to dissolve the furoxans did not influence bacterial growth.
198 Of the three compounds, only KN455 provided a reproducible inhibition growth halo (12.3 ± 0.57
199 mm). In order to investigate the possible involvement of nitric oxide (NO) in the observed inhibitory
200 activity, the corresponding furazan CF389, which is known not to release NO, was included in the
201 analysis and no inhibition halo was observed (Figure 2). Furthermore, the corresponding 4-nitro
202 isomer (KN454) was assayed to evaluate whether the nitro position on the heteroring influenced
203 antimicrobial activity. In fact, KN454 failed to show any antimicrobial activity (Figure 2) and 3-nitro-
204 4-phenylfuroxan was the only active compound. Furthermore, its activity was dose-dependent (data
205 not shown).

206 The MIC value of KN455 was 125 μ M, and the MBC value 250 μ M, meaning that its Killing
207 Quotient, calculated as MBC/MIC ratio, was lower than 4. This would indicate that the effect of
208 KN455 is bactericidal. The MIC value of its isomer KN454 was 1 mM, whereas the MIC values of
209 furoxans CF357 and CF1656, and furazan CF389 were all higher than 2 mM.

210 Confocal analyses were performed to evaluate the effect of KN455 on suspended PAO1 cells.
211 The PAO1 strain was transformed with the Green Fluorescent Protein-gene-carrying plasmid, pVO,
212 under the control of the *araBAD* promoter. Upon induction with arabinose, the cultures produced
213 enough GFP to give a fluorescent signal, while cells that were not induced with arabinose were not
214 fluorescent (data not shown). Upon 24 hours of treatment with KN455 125 μ M (MIC), few survivors
215 were detected and most showed morphological changes compared to untreated (not shown) and
216 DMSO 0.5 % treated cells (3A). As seen in Figure 3B, the surviving bacteria are longer than the
217 untreated cells. In fact, the binary division process seems to be impaired, septation appears to be
218 incomplete and giant cells are present in the treated group. Moreover, some of the filamentous rods
219 show bright fluorescence while others show less pronounced GFP fluorescence. CF389 (Figure 3C)
220 and KN454 125 μ M (data not shown) did not impaired the cellular viability as KN455, however few
221 long cells were detectable. The administration of CF1556 (Figure 3D) and CF357 (data not shown)
222 did not cause any change in cellular density, GFP expression and bacterial morphology.

223 ***Effect of furoxans on the inhibition of *P. aeruginosa* biofilm formation***

224 As it is known that furoxans release NO, the effect of increasing furoxan concentration (5, 50
225 and 500 μ M) on PAO1 biofilm formation was investigated. Compounds were added to minimal
226 medium together with PAO1 and the adherent biomass was examined using crystal violet staining
227 after 6 hours of incubation. Compounds were dissolved in DMSO 0.5 %, without significantly
228 changing the amount of adherent biomass ($p = 0.61$) compared to untreated controls (data not shown).

229 The nitro substituted furoxan, KN455, provided the most significant and dose-dependent biofilm
230 inhibition of the three different furoxans tested, giving significant inhibition rates at the highest
231 concentrations of 50 and 500 μ M, respectively (Figure 4A). The CN substituted furoxan, CF357,
232 inhibited biofilm formation in a significant manner ($p = 0.014$) at 500 μ M (Figure 4B), while the
233 CONH₂ substituted analogue, CF1656, did not show any anti-biofilm effect at any tested
234 concentration (Figure 4C). As the nitro derivative showed the highest inhibitory activity, its 4-nitro
235 isomer KN454 and the corresponding des-NO furazan analogue, CF389, were also tested for their
236 ability to inhibit biofilm formation. The rates of inhibition caused by KN454 and CF389 at the highest
237 tested concentrations were statistically significant as shown in figures 4D and 4E, respectively (data
238 not shown).

239 The viability of sessile biofilm cells was checked and KN455, alone among the tested furoxans,
240 displayed a toxic effect against this sub-population (Figure 5A). A ~3-log unit decrease, compared to
241 untreated cells, was observed. The corresponding 4-nitro isomer, KN454, and furazan, CF389, caused
242 2-log unit decreases when administered at 500 μ M.

243 The planktonic counterparts of each biofilm were also evaluated as they play an important role
244 in bacterial dissemination and equilibrium maintenance in the biofilm community. DMSO 0.5 % did
245 not influence the cellular viability of planktonic biomass, compared to untreated cells (data not
246 shown), while KN455 provided a much more significant decrease in viable cells ($p = 3.2 \times 10^{-7}$) than
247 the untreated samples (Figure 5B). Furoxans, CF357 and CF1656, did not alter the viability rate of
248 cells (Figure 5B). Furazan CF389 and furoxan KN454 only caused a 1-log unit decrease, compared
249 to untreated control biofilms.

250 Confocal analyses of 24- and 48-hour-old biofilms showed that the inhibitory effect of KN455
251 500 μ M was also reliable when used in prolonged treatment (Figure 6). The GFP signal of a furoxan-
252 incubated biofilm was lower than those of untreated and DMSO 0.5 % v/v treated biofilms. KN455's
253 inhibitory effect was also evident against a 48-hour-old biofilm, which was noticeably thicker than
254 the 24-hour-old biofilm (Figure 6). The corresponding furazan CF389 was not as effective as KN455,
255 especially against 48 hour old biofilm (figure 6).

256 ***Effect of furoxans on biofilm eradication***

257 While the inhibition of biofilm formation is an important goal, biofilm eradication is much more
258 difficult to achieve and antimicrobials usually fail. The compounds were administered at 500 μ M for
259 1h to cells grown for 5h to assay biofilm eradication power. The solvent was not toxic to cells (data
260 not shown). KN455 showed the most statistically significant ($p = 9.65 \times 10^{-4}$) eradication power, of
261 the furoxans tested (Figure 7). The decreases in adherent biomass caused by isomer KN454 and des-

262 NO furazan CF389 were not significant. The solvent was not toxic to cells in tests on planktonic
263 counterparts and the biomass changes observed upon furoxan treatment were not significant (data not
264 shown), compared to control samples DMSO treated biofilms.

265

266 *Insight into anti-Pseudomonas activity of KN455*

267 KN455 was the most effective anti-*Pseudomonas* agent of the furoxans tested as it inhibited
268 both cellular growth and biofilm formation, and even showed moderate biofilm eradicating power.
269 The kinetics of NO_x release from KN455 were compared to those of the other furoxans, CF357 and
270 CF1656, because it this thought that NO may play the role of anti-biofilm messenger. Furthermore,
271 the isomer KN454 and corresponding furazan CF389, which were included in the antimicrobial and
272 antibiofilm assays, are also included in Figure 8, as % mol/mol. The amounts of NO₂⁻ detected for 3-
273 nitro-4-phenylfuroxan (KN455) were about 130 mol/mol after 1 hour. The amount of NO₂⁻ produced
274 by KN455 is the sum of the NO₂⁻ formed by the nucleophilic substitution of the nitro group (see
275 supporting information, S1, pathway a), and of the NO₂⁻ produced by the aerobic oxidation of the NO
276 released following the ring opening of the tetrahedral intermediate precursors (see supporting
277 information, S1, pathways b, R=NO₂, R = CyS). DAN (2,3-diaminonaphtalene) assays (Fang *et al.*
278 2009), which allow NO centre dots to be specifically detected, were carried out to shed further light
279 onto this process. NO₂⁻ was the dominant species produced by KN455 after 1 h, whereas NO only
280 corresponded to 5% of total NO_x. The amount of NO₂⁻ detected for the 4-nitro isomer KN454 was
281 about 80 % mol/mol after 1 hour (Figure 8). For the *des*NO-nitrofurazan CF389, which cannot release
282 NO from the heterocycle core, the reaction rate was slower and the amount of NO₂⁻ detected was 25
283 % mol/mol after 1 hour (Figure 8). 3-cyanofuroxan, CF357, and 3-carbamoylfuroxan, CF1656,
284 produced nitrite in good and modest amounts, respectively. However, it is worth noting that these
285 yields derived exclusively from the oxidation of NO (DAN test).

286 The highest rate of NO release was displayed by CF357, which, however, showed low anti-
287 biofilm activity. Furoxans KN455 and CF1656, which release the same very low NO amount, showed
288 different effects on microbial growth and biofilm formation. These data seem to rule out the possible
289 involvement of NO in KN455 toxicity.

290 In order to investigate this hypothesis, biofilm formation experiments were repeated in the
291 presence of PTIO, a known NO radical scavenger, and no changes were observed (data not shown).
292 The possible involvement of nitrite, the other species released in large amounts by KN455, was also
293 evaluated. The biofilm inhibition effect induced by acidified sodium nitrite was checked and
294 compared to that of equimolar increasing concentrations of KN455. Sodium nitrite was less active
295 than KN455 at the tested concentrations (Figure 9).

296 Since previous studies have shown that thiol groups, such as those present in L-cysteine (L-
297 cys), can promote the liberation of NO_x (NO and NO₂⁻) from furoxan compounds (Feelisch M. *et al.*
298 1992), PAO1 was treated with increasing concentrations of KN455 in the presence of an excess of
299 cysteine 5mM. L-Cysteine decreased the anti-biofilm activity of 50 μM and 500 μM KN455 (Figure
300 10). Furthermore, L-cysteine abolished the anti-biofilm activity of KN455-related compounds,
301 KN454 and CF389 (data not shown).

302 The reaction between L-cysteine and KN455 was monitored by HPLC in order to more deeply
303 probe the role played by thiol groups. HPLC/mass data (Figure S2) indicate that, after 20 min, the
304 compound was almost quantitatively transformed into 3-cysteinyl-4-phenylfuroxan (282 M+1, 120
305 M-cysteinyl), which is the product of the nucleophilic substitution of the 3-nitro group with cysteine
306 (see supporting information S1, pathway a). Over time, this latter compound was largely transformed
307 into unidentified products and NO₂⁻ (see supporting information S1, pathway b). The 4-nitro isomer,
308 KN454, also reacted with L-cysteine giving rise to the corresponding cysteinyl derivatives (see
309 supporting information S3). Des-NO furazan CF389, which cannot release NO from its heterocycle
310 core, behaved similarly and afforded the cysteinyl substituted product. 3-Cyanofuroxan, CF357, and
311 3-carbamoylfuroxan, CF1656, produced nitrite and unidentified products (see supporting information
312 S4).

313 Discussion

314 Previous studies have shown that thiol groups, such as those present in L-cysteine (L-cys), can
315 promote the liberation of NO_x from furoxan compounds and increase their activity [18]. In this study,
316 three phenylfuroxan derivatives that can release varying amounts of NO_x were assayed for their
317 antimicrobial activity against *P. aeruginosa* PAO1, and it would appear that the substituent influences
318 furoxan antimicrobial activity; the NO₂-substituted analogue, KN455, inhibited the growth of PAO1,
319 whereas CN- and CONH₂-substituted furoxans did not show any antimicrobial activity in the solid
320 media. Furthermore, KN455 activity was bactericidal. Interestingly, the position of the nitro group
321 on the furoxan skeleton significantly influences antimicrobial activity against *P. aeruginosa* PAO1;
322 KN454 did not show any inhibition halo in the solid media and its MIC value was four-times higher
323 than that of KN455.

324 Furthermore, the chosen furoxans showed different NO_x release rates. As NO is emerging as
325 an anti-biofilm messenger [32], the biofilm formation inhibition activity of the furoxans was
326 investigated and correlated with NO_x release. KN455, which is characterized by very low NO release,
327 proved itself to be the most active compound in inhibiting biofilm formation, whereas CF357 and

CF1656, which can release good and very low amounts of NO, respectively, showed weak or null activity even at the highest concentrations tested. CF389, which is known not to release NO, and KN454, which releases nitrite, showed inhibitory activity. It would thus appear that nitric oxide does not play a role in furoxan biofilm inhibition. Indeed, KN455 was very toxic and no viable cells were recovered from the planktonic subpopulation of the six-hour-old biofilms. By contrast, the 4-nitro isomer KN454 was not toxic to the planktonic subpopulation, and only caused a slight decrease in cellular viability. The two furoxans showed different selectivities against biofilm cellular subpopulations; KN455 inhibited the growth of both planktonic and adherent cells, while KN454 was selectively active against adherent bacteria.

Furthermore, the furoxans release different amounts of nitrite (KN455 > CF357 > CF1656), which has been reported to inhibit staphylococcal biofilm formation [33, 34]. Under the tested conditions, sodium nitrite alone did inhibit *P. aeruginosa* PAO1 biofilm formation, but showed lower activity than equimolar concentrations of KN455. Although the nitrite-effect cannot be ruled out, other mechanisms should be considered.

L-cysteine prevented KN455 from inhibiting biofilm formation. It is reasonable to connect KN455 activity with its capacity to react with the thiol groups (or other nucleophilic centres) present on functional targets that are involved in cellular division and/or biofilm formation. This may also explain the activity of the 4-nitro isomer, KN454, and of the related des-NO furazan, CF389, which behave similarly in their reactions with cysteine.

Cysteine has also been demonstrated to act as an antioxidant, supporting the hypothesis that furoxan and furazan can elicit oxidative stress [35]. This is in agreement with the morphological changes observed upon furoxan treatment. Indeed, filamentation is a typical stress response that occurs when cell growth continues in the absence of cell division [36]. Both highly fluorescent giant cells and other cells that showed less pronounced GFP fluorescence were observed in furoxan/furazan-treated samples. GFP expression in the first group of cells was compatible with functional transcriptomic and expression machinery. The longest bacteria showed fuzzy fluorescence, which is compatible with GFP that has been broken down following decreased protein synthesis and/or permeability stress. However, the impairment of divisome machinery and cell wall septation seems to precede a pre-death phase. The low fluorescence of filamentous cells can probably be related to decreased metabolic activity before cellular death, as the formation of multinucleate giant cells may be a pre-death phase. Both nitro-furoxans, KN455 and KN454, and furazan, CF389, seem to elicit cellular stress, which induces cellular elongation.

360 In conclusion, nitrofuroxan KN455 quite potently inhibits *P. aeruginosa* PAO1 growth and
361 prevents PAO1 biofilm formation. This activity does not appear to be related to its capacity to release
362 small amounts of nitric oxide, rather than nitrite. Oxidative stress and/or the impairment of divisome
363 machinery, which is ascribable to its strong electrophilic properties, may contribute to this anti-
364 *Pseudomonas* activity. Additional work is necessary to investigate the intriguing differences found
365 between furoxan KN455 and the isomer KN454, as well as to identify their target/s.

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370 **Disclosure statement**

371 The authors report no potential conflict of interest.

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470

471 CAPTIONS

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473

474 **Figure 1.** Schematic representation of the furoxans and furazan investigated.

475 **Figure 2.** Kirby-Bauer disk diffusion tests. Effect of 20 µl of DMSO 0.5 %, KN455, KN454 and
 476 CF389 at a concentration of 50 mM on the growth of *P. aeruginosa* PAO1.

477 **Figure 3.** Confocal images of *P. aeruginosa* PAO1 that was GFP-tagged after 24 hours of treatment
 478 with DMSO 0.5 % (A) and KN455 (B), CF389 (C) and CF1656 (D) 125 µM, respectively (bar 10
 479 µm). GFP expression was induced by arabinose 1 mM, one hour before confocal analyses.

480 **Figure 4.** Effect of phenyl-R-substituted furoxans/furazan on the inhibition of *P. aeruginosa* PAO1
 481 biofilm formation. The effect was evaluated by CV staining OD595 values upon treatment for 6 hours
 482 with KN455 (A), CF357 (B), CF1656 (C), KN454 (D) and CF389 (E) at increasing concentrations,
 483 with bars representing data from at least three biological replicates and error bars representing
 484 standard deviation from the mean *, $p \leq 0.05$; **, $p \leq 0.001$.

485 **Figure 5.** Viable counts of the adherent (A) and planktonic (B) phases upon the inhibition of *P.*
 486 *aeruginosa* PAO1 biofilm formation exerted by furoxans/furazan 500 µM. The data expressed as
 487 CFU well⁻¹ and CFU ml⁻¹ of survivors, respectively, are the means of at least three independent
 488 experiments \pm SD * $p < 0.05$, ** $p < 0.001$ and *** $p < 10^{-6}$.

489 **Figure 6.** Projections and sections of CLSM images of *P. aeruginosa* PAO1 GFP-tagged biofilms
 490 that were subjected to furoxan/furazan and solvent treatments. Bacteria were treated for 24 hours
 491 (left) or 48 hours (right) with KN455 and CF389 500 µM or DMSO 0.5 %. The live cells that
 492 express GFP are green.

493 **Figure 7.** *P. aeruginosa* biofilm dispersal upon treatment with furoxans at 500 µM for 1h. The bars
 494 represent data from three biological replicates and error bars represent the standard deviation from
 495 the mean *, $p \leq 0.05$.

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497 **Figure 8.** NO and total NO₂⁻ released by furoxans (KN455, KN454, CF357,CF1656) and furazan
498 CF389 in the presence of L-cysteine (5:1 molar excess) at a selected time point (60 min). The results
499 are expressed as percentage (% mol/mol) of NO or total NO₂⁻ released with respect to the quantity of
500 parent compound. Bars represent data from three or more replicates and error bars represent standard
501 deviations from the mean.

502 **Figure 9.** *P. aeruginosa* biofilm inhibition upon treatment with KN455 and Sodium Nitrite for 6
503 hours at increasing concentrations. The error bars represent the standard deviation from the mean of
504 three biological replicates.

505 **Figure 10.** *P. aeruginosa* biofilm inhibition upon treatment with KN455 500 µM for 6 hours in the
506 presence and absence of L-cysteine 5 mM. The bars represent OD595 values upon Crystal Violet
507 staining of three biological replicates, whereas error bars represent the standard deviation from the
508 mean.

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